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This amendment adds, changes and/or deletes claims in this application. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claim(s) remain under examination in the application, is presented, with an appropriate defined status identifier.

After amending the claims as set forth above, claims 1-2, 4-6 and 8-10 are now pending in this application.

I. Request for Reconsideration of Restriction Requirement

As a predicate to submission of a petition pursuant to 37 CFR §§1.144 and 1.181, Applicant hereby requests that the requirement for election between SEQ ID Nos. 2, 4, 6 and 8 within the claims of Group I (Claims 1-10). Reconsideration and examination of the species represented by SEQ ID Nos. 2, 4, 6 and 8 together within Group I is requested for the following reasons.

Although the sequences in question are amino acid sequences, the Examiner correctly notes that they are amino acid sequences encoded by different genes. The Examiner contends, however, that the Examination Guidelines allowing up to 10 different and distinct nucleotide sequences to be examined without restriction are not binding. Applicant respectfully disagrees.

As reflected in MPEP §803.4, the Commissioner waived the provisions of 37 CFR 1.141 to allow more than one sequence to be examined in a single application, notwithstanding the fact that the sequences may be regarded as extending to independent and distinct inventions. This waiver has *not* been withdrawn or modified, and is still in effect:

> Nucleotide sequences encoding different proteins are structurally distinct chemical compounds and are unrelated to one another. These sequences are thus deemed to normally constitute independent and distinct inventions within the meaning of 35 U.S.C. 121....Nevertheless, to further aid the biotechnology industry in protecting its intellectual property without creating an undue burden on the Office, **the Commissioner has decided *sua***

> ***sponte* to partially waive the requirements of 37 CFR 1.141 *et seq.* and permit a reasonable number of such nucleotide sequences to be claimed in a single application.** See *Examination of Patent Applications Containing Nucleotide Sequences,* 1192 O.G. 68 (November 19, 1996).
>
> It has been determined that normally ten sequences constitute a reasonable number for examination purposes. Accordingly, in most cases, up to ten independent and distinct nucleotide sequences will be examined in a single application without restriction.
>
> (*Manual of Patent Examination Procedure*, Section 803.04, **2003 edition**; emphasis added)

Only rarely are exceptions to the determination that 10 sequences is a reasonable number allowed; e.g., "In some exceptional cases, the complex nature of the claimed material, for example a protein amino acid sequence reciting three dimensional folds, may necessitate that the reasonable number of sequences to be selected be less than ten." *Id.*

Based on the foregoing, Applicant respectfully submits that the foregoing procedure allowing for up to 10 sequences to be examined without restriction in a single application is still in force, such that the Commissioner's ruling and waiver govern examination of this application.

As acknowledged, SEQ.ID.Nos. 2, 4, 6 and 8 are all directed to the same mutation, as applied to individual members of the NGF family of neurotrophins. The NGF family of neurotrophins are substantially homologous, target the same receptors, and share substantially similar overall bioactivity. *See, e.g.,* Ebendal, *J.Neurosci.Rsch.*, 32:461-470 (1992)(review paper providing an overview of the activity of NGF family neurotrophins and the distribution of their receptors; copy enclosed for ease of reference). The claimed sequences are not, therefore, the kind of "complex material" whose claiming within a single application justifies restriction under MPEP §803.04. Reconsideration and withdrawal of the final restriction requirement is therefore requested.

As a further basis for reconsideration, Applicant notes that Claim 1 has been amended to recite the targeted NGF family of proteins as members of a Markush grouping. Restriction

practice relating to Markush groupings requires that the members of the group be examined together so long as they are relatively few in number *or* closely related. MPEP §803.02. Applicant submits that either or both of these conditions are present here, such that the members of the NGF family recited in Claim 1 with the Markush terminology "selected from the group" must be examined together in this application.

For all of the foregoing reasons, reconsideration and withdrawal of the restriction requirement is requested.

II. Support for Amendments

Claim 1 has been amended to incorporate the limitations of Claim 3, now cancelled. Claim 5 has been amended to incorporate the limitations of Claim 7, now cancelled. No new matter is added to the application by these amendments.

Further, the Specification is amended to correct an obvious typographical error. At page 7, lines 4-5, the start of the mature protein is identified as beginning 4 amino acids downstream of the underlined amino acid at position 76. Four amino acids downstream of the amino acid at position 76 would inherently be the residue at position 80, not 81 (see, sequence recited at page 7, lines 6-10). The Specification has been amended to correct this obvious typographical error; no new matter has been added as a result of the amendment.

III. Response to Objections Under 35 USC §112, Second Paragraph.

Claims 1-10 are objected to for indefiniteness in various respects.

First, the phrase "at a position 8 amino acids upstream from the site of cleavage for the mature growth factor" is objected to as being unclear with respect to the site of cleavage. Applicant respectfully disagrees.

As discussed in the Specification, the N-glycosylation site targeted for substitution by the invention is preserved throughout all known neurotrophin-family pro-neurotrophins. *See,*

Specification, page 5, lines 14-20. The sites for cleavage of the precursor sequences within this family are known and publicly accessible through databases such as GENBANK. *See,* Specification at page 7, lines 13-16. Moreover, the actual sites of cleavage for all molecules recited in the claims (as well as their corresponding coding and substituted sequences) are explicitly set forth in the Specification. *See,* page 5, line 21 through page 8, line 50.

Thus, Applicant submits that the identity of the recited cleavage sites is amply and unambiguously defined in the Specification. Reconsideration and withdrawal of the objections under §112, second paragraph with respect to the definition of the sites of cleavage is therefore requested.

Claim 9 is also objected to on the basis that the language lacks clarity with respect to whether the claimed protein consists of or comprises the recited sequences.

Applicant respectfully submits that Claim 9 is in proper Markush format ("X protein selected from the group of such proteins consisting of A, B and C") and, as such, requires neither the suggested "comprising" or "consisting essentially of" language. Reconsideration and withdrawal of the objection to Claim 9 is therefore requested.

IV. Response to Claim Rejections Under 35 USC §112, First Paragraph.

Claims 1-8 and 10 are rejected as not being enabled by the disclosure and/or for lack of written description. Applicant respectfully disagrees.

The §112, first paragraph rejections are based on the perception that the neurotrophin family targeted by the claims includes more than the members of the NGF family. Office Action, page 5, first paragraph. This perception is inaccurate. As defined for purposes of this invention, the "family of neurotrophins" referred to in the Action is defined by the text in the Specification age page 1, lines 21 through 32. The proteins further listed on page 2, at lines 1-10, are explicitly defined for purposes of this invention as "other nervous system growth factors".

To further clarify this distinction, Markush groupings of the neurotrophins included within the scope of the present claims, as amended, are recited. Reconsideration and withdrawal of the claim rejections is therefore respectfully requested.

CONCLUSION

Applicant believes that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 50-0872. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 50-0872.

If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 50-0872.

Respectfully submitted,

Date 1-8-04

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Journal of Neuroscience Research

Volume 32 • Number 4 • August 1992

WILEY-LISS

ISSN 0360-4012

Journal of Neuroscience Research 32:461–470 (1992)

Review

Function and Evolution in the NGF Family and Its Receptors

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The gene family of neurotrophins includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). Recently, neurotrophin-5 (NT-5), a possible mammalian homologue to NT-4 described in the frog *Xenopus,* has been cloned in man and rat. The neurotrophins stimulate survival and differentiation of a range of target neurons by binding to cell surface receptors. The structure of NGF has recently been clarified from crystallographic data. The similarities between the different neurotrophins are substantial with the variable regions, giving specificity to each of the family members, being localized to some exposed loop regions. Low-affinity binding (K_d of 10^{-9} M) of all tested neurotrophins is mediated via a 75 K glycoprotein (LNGFR) that has been cloned and characterized. A 140 K tyrosine protein kinase encoded by the proto-oncogene *trk* has been found to bind NGF with high affinity (K_d of 10^{-11} M) and to evoke the cellular neurotrophic responses. In addition, a protein encoded by the *trk*-related gene *trk*B has been shown to bind BDNF. Recently, a third member of the *trk* family, *trk*C, has been cloned and demonstrated to function as a high-affinity receptor for NT-3. The expression of *trk* and LNGFR mRNA are co-localized in the rat brain to the medial septal nucleus and the nucleus of Broca's diagonal band containing the NGF-responsive magnocellular cholinergic neurons projecting to hippocampus and cerebral cortex. In sharp contrast, the pattern of expression of *trk*B is widely spread in many areas of the cortex as well as lateral septum. The *trk*B protein might serve general functions in large areas of the cortex. Site-directed mutagenesis and expression of recombinant chimaeric neurotrophin proteins have made it possible to localize a likely region for the interaction between NGF and the LNGFR. This region could be altered, resulting in the total loss of LNGFR binding by the mutant NGF protein without affecting the binding to the *trk* receptor which was sufficient for the full biological activity. Cladistic analysis of likely phylogenies within the neurotrophins shows BDNF and NT-4 to be most closely related whereas NGF may be the sister group to NT-3, BDNF, and NT-4. Neurotrophins offer obvious clinical possibilities for treatment of neurodegenerative diseases.

Key words: neurotrophins, nerve growth factor receptor, proto-oncogene *trk*, molecular evolution, cholinergic neurons, medial septal nucleus, in situ hybridization

INTRODUCTION

Substantial progress has recently been made in research on nerve growth factor (NGF) and the other members of the gene family of neurotrophic factors (the neurotrophins). Apart from NGF, the neurotrophins include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), and neurotrophin-5 (NT-5). These factors are produced in limiting amounts in the target tissues and mediate the cell interactions regulating neuron survival during the period of naturally occurring neuronal death in development. The release of these proteins is believed to regulate not only the survival of neurons but also the extent of innervation of the target tissues. As well as being important in neuronal development, neurotrophic factors also have a function in the adult nervous system.

THE MEMBERS OF THE NGF GENE FAMILY

The best known neurotrophic factor is β-nerve growth factor (NGF), a basic 118 amino acid protein

Received March 25, 1992; revised April 22, 1992; accepted April 23, 1992.

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which acts on many sensory and sympathetic neurons in the peripheral nervous system (Levi-Montalcini, 1987). Nerve growth factor is also present in the brain where it serves a trophic function in the development and maintenance of cholinergic neurons of the basal forebrain (review by Ebendal, 1989).

The NGF protein has been purified and sequenced (Angeletti and Bradshaw, 1971; Angeletti et al., 1973) from the submandibular gland of the male mouse. The amino acid sequence has been confirmed by analysis of complementary DNA (cDNA) and genomic clones for NGF. Nerve growth factor is synthesized as a 305 amino acid long prepro-NGF (Scott et al., 1983; Ullrich et al., 1983; Ebendal et al., 1986; Selby et al., 1987a; Whittemore et al., 1988). The mature NGF protein is generated by proteolytic cleavage at dibasic amino acid residues in the prepro-NGF.

The cloning of NGF (Scott et al., 1983; Ullrich et al., 1983; Ebendal et al., 1986; Meier et al., 1986; Selby et al., 1987a,b; Fahnestock and Bell, 1988; Whittemore et al., 1988; Schwarz et al., 1989; Carriero et al., 1991) has allowed the deduction of the sequence for the mature NGF protein from nine different species (mouse, rat, the African rat *Mastomys natalensis*, guinea pig, human, bovine, chicken, cobra, the clawed toad *Xenopus*). The similarity in amino sequence among NGF from different species (some shown in Fig. 1) is high (66–98%) with the variable regions located in a few hydrophilic domains (Meier et al., 1986; Whittemore et al., 1988; Ebendal et al., 1989).

A second protein with neurotrophic activities reminiscent of but not identical to NGF was isolated from the pig brain and termed brain-derived neurotrophic factor (BDNF; Barde et al., 1982). BDNF also supports the survival of neural crest-derived embryonic sensory neurons in vitro (Barde et al., 1982; Leibrock et al., 1989). However, unlike NGF, BDNF also supports placode-derived neurons from the nodose ganglion (Davies and Lindsay, 1986). Regulation of neuronal survival in the brain by BDNF remains to be demonstrated.

A genomic clone isolated for the porcine BDNF led to the finding that the mature BDNF and NGF proteins show striking amino acid similarities (Leibrock et al., 1989; Fig. 1). BDNF is expressed mainly in CNS and is only detectable in heart, lung, and skeletal muscle in the periphery. During development BDNF is expressed in the brain at initially low levels but later increases to become the most widespread neurotrophin in different areas of the brain. BDNF has been cloned from pig, mouse, rat, and human (Leibrock et al., 1989; Hofer et al., 1990; Maisonpierre et al., 1991). Partial sequence is known also from chicken and *Xenopus* (Hallböök et al., 1991; Isackson et al., 1991).

Based on cloning strategies utilizing the partial sequence similarities between NGF and BDNF, a third member of the NGF family named neurotrophin-3 (NT-3) was isolated (Rosenthal et al., 1990; Maisonpierre et al., 1990; Ernfors et al., 1990; Kaisho et al., 1990). The DNA clones isolated from rat, mouse, and human encode a protein having a strong sequence similarity to both NGF and BDNF (Fig. 1). The distribution of NT-3 mRNA in the adult rat brain displays a high degree of regional specificity. It is predominantly expressed in a subset of pyramidal and granular neurons in the hippocampus (Ernfors et al., 1990). Very weak expression may also be observed in the cerebellum and cerebral cortex. BDNF mRNA is more widely distributed in the rat brain, although hippocampus also contains the highest amount, followed by cerebral cortex, pons, and cerebellum. In situ hybridization histochemistry shows this factor to have a neuronal localization partly distinct from that of NGF and NT-3 (Wetmore et al., 1990). Interestingly, these three neurotrophic proteins are maximally expressed at different times of brain development in the rat with a peak of NT-3 mRNA shortly after birth, BDNF mRNA around 2 weeks, and NGF mRNA 3 weeks after birth (Whittemore et al., 1986; Ernfors et al., 1990). It has recently been shown that the peak in NT-3 mRNA expression after birth is partly due to a transient expression of NT-3 mRNA in the cingulate cortex (Friedman et al., 1991).

Further use of the conserved regions of NGF, BDNF, and NT-3 for PCR searches of additional members of the NGF gene family has recently resulted in the identification of neurotrophin-4 (NT-4), described by full length sequence in the clawed toad *Xenopus* (Hallböök et al., 1991). The expression of NT-4 in *Xenopus* tissues is restricted to the ovary. The biological activity of recombinant *Xenopus* NT-4 protein is similar to that of BDNF with stimulation of sensory neurons in culture (Hallböök et al., 1991). Subsequently, mammalian NT-4-like sequences have been isolated. Ip et al. (1992) isolated NT-4 clones from the rat and human genome using a specific *Xenopus* NT-4 sequence for the design of a downstream PCR primer combined with an upstream primer encoding a sequence shared by all known neurotrophins. In addition to the active human NT-4 gene, Ip et al. (1992) describe a NT-4 pseudogene with several frameshifts, an internal stop codon, lack of two of the essential cysteine residues, as well as a lack of the cleavage site used to process the mature neurotrophins. This is the first description of a pseudogene within the neurotrophin gene family. Berkemeier et al. (1991) used a slightly different PCR strategy, involving the use of degenerate primers encoding sequences shared by NGF, BDNF, and NT-3 in the search for additional neurotrophins in the human genome. They found DNA fragments encoding a protein, designated neurotrophin-5 (NT-5) by these authors, with the same sequence as the human NT-4 described by Ip et al. (1992).

```
                                                        ---- I ----    --- II ---
                                                         β loop         β loop
NGF                              *
  rat      +1  S--STHPVF-HMGEFSVCDSVSVWVG--DKTTATDIKGKEVTVLGEVNINNS-VFKQYFF
  human    +1  SS-S-HPIF-HRGEFSVCDSVSVWVG--DKTTATDIKGKEVMVLGEVNINNS-VFKQYFF
  chicken  +1  T-A--HPVL-HRGEFSVCDSVSMWVG--DKTTATDIKGKEVTVLGEVNINNN-VFKQYFF
NT-3
  rat      +1  Y--AEHKS--HRGEYSVCDSESLWVT--DKSSAIDIRGHQVTVLGEIKTGNSPV-KQYFY
  human    +1  Y--AEHKS--HRGEYSVCDSESLWVT--DKSSAIDIRGHQVTVLGEIKTGNSPV-KQYFY
BDNF
  rat      +1  H--SD-PA--RRGELSVCDSISEWVTAADKKTAVDMSGGTVTVLEKVPVSKG-QLKQYFY
  human    +1  H--SD-PA--RRGELSVCDSISEWVTAADKKTAVDMSGGTVTVLEKVPVSKG-QLKQYFY
NT-4/NT-5
  Xenopus  +1  ASGSDSVSLSRRGELSVCDSVNVWVT--DKRTAVDDRGKIVTVMSEIQTLTG-PLKQYFF
  human    +1  -GVSETAPASRRGELAVCDAVSGWVT--DRRTAVDLRGREVEVLGEVPAAGGSPLRQYFF


                  ---- III ----                      -- IV --   - V -
                   reverse turn                                 β loop
NGF             *              *          *
  rat      +55  ETKCRA-------PNPVESGCRGIDSKHWNSYCTTTHTFVKALTTDD-KQAAWRFIRIDT
  human    +55  ETKCRD-------PNPVDSGCRGIDSKHWNSYCTTTHTFVKALTMDG-KQAAWRFIRIDT
  chicken  +54  ETKCRD-------PRPVSSGCRGIDAKHWNSYCTTTHTFVKALTMEG-KQAAWRFIRIDT
NT-3
  rat      +54  ETRCKE-------ARPVKNGCRGIDDKHWNSQCKTSQTYVRALTSENNKLVGWRWIRIDT
  human    +54  ETRCKE-------ARPVKNGCRGIDDKHWNSQCKTSQTYVRALTSENNKLVGWRWIRIDT
BDNF
  rat      +55  ETKCNP-------MGYTKEGCRGIDKRHWNSQCRTTQSYVRALTMDSKKRIGWRFIRIDT
  human    +55  ETKCNP-------MGYTKEGCRGIDKRHWNSQCRTTQSYVRALTMDSKKRIGWRFIRIDT
NT-4/NT-5
  Xenopus  +58  ETKCNP-------SGSTTRGCRGVDKKQWISECKAKQSYVRALTIDANKLVGWRWIRIDT
  human    +58  ETRCKADNAEEGGPGAGGGGCRGVDRRHWVSECKAKQSYVRALTADAQGRVGWRWIRIDT


NGF                * *
  rat      +107  ACVCVLSRKAARRG   +120
  human    +107  ACVCVLSRKAVRRA   +120
  chicken  +106  ACVCVLSRKS-GRP   +118
NT-3
  rat      +107  SCVCALSRK-IGRT   +119
  human    +107  SCVCALSRK-IGRT   +119
BDNF
  rat      +108  SCVCTLTIK-RGR-   +119
  human    +108  SCVCTLTIK-RGR-   +119
NT-4/NT-5
  Xenopus  +111  ACVCTLLSRT-GRT   +123
  human    +118  ACVCTLLSRT-GRA   +130
```

Fig. 1. Comparison of the amino acid sequences for the members of the NGF family of neurotrophic factors, the neurotrophins. The mature protein sequences are shown aligned using the one-letter code for the amino acids (see references in legend to Figure 5). Gaps have been introduced to reach best fit. Amino-acid residues strictly conserved among the sequences shown are indicated by bold face type. The six cysteine residues, forming three intramolecular bonds, common to all known neurotrophins, are marked by asterisks. The *Xenopus* NT-4 sequence is from Hallböök et al. (1991) and the related human sequence is from Berkemeier et al. (1991), described as neurotrophin-5, and from Ip et al. (1992), described as human NT-4. The regions corresponding to β hairpin loops and the single region forming reverse turns in the NGF protein (McDonald et al., 1991) are five variable regions (I to V) studied by site-directed mutagenesis in the NGF protein and by the construction of chimeric NGF-BDNF molecules (Ibáñez et al., 1990, 1991, 1992).

The amino acid sequences for *Xenopus* NT-4 and for the human NT-4/NT-5 are shown in Figure 1 aligned with the other neurotrophins. A peculiarity is the extra seven amino acids inserted in positions 64 to 70 in the mature mammalian NT-4/NT-5. Another peculiar feature is that the pre-pro sequence is some 50 amino acids shorter than in the other known neurotrophins (Berkemeier et al., 1991; Ip et al., 1992). The human and rat prepro NT-4/NT-5 show 91% amino acid similarity. The human mature NT-4/NT-5, a predicted protein of 123 amino acids, has 66% similarity to *Xenopus* NT-4 and 50% similarity to NGF (Berkemeier et al., 1991). Berkemeier et al. (1991) argue that NT-5 is distinct from NT-4 because it is expressed at low levels in several peripheral organs and is able to stimulate sympathetic neurons when expressed as a recombinant protein, properties not shared with *Xenopus* NT-4. Clearly, more studies are needed to reach an understanding of the relation among the different neurotrophins of the NT-4/NT-5 group, their tissue specific and developmental expression, and their physiological functions.

From studies of the recombinant neurotrophins it is evident that although the members of the NGF family share considerable sequence similarities (Fig. 1), NGF, BDNF, and NT-3 each have unique biological activities (Ernfors et al., 1990; Ibáñez et al., 1991) and may cooperate to support the development and maintenance of the vertebrate nervous system. The final number of neurotrophins evolved in such a network of cell interactions functioning in brain development remains to be determined.

MOLECULAR STRUCTURE OF NGF

The structure of NGF has recently been clarified from crystallographic data (McDonald et al., 1991) and comprises seven β-strands forming three antiparallel pairs (Fig. 2). The amino acids which are important for establishing this configuration are well conserved among the different neurotrophins, which argues strongly for very similar three-dimensional structures in BDNF, NT-3, and NT-4/NT-5. The variable amino acid residues are located primarily in four regions: three β-hairpin loops (residues 29–35, 43–48, and 92–98; Figs. 1 and 2) and one region comprising three consecutive reverse turns (residues 58–68; Figs. 1 and 2). These regions have earlier been noted due to their hydrophilic nature (Meier et al., 1986; Ebendal et al., 1989) and are well conserved among NGF proteins from different species (Ebendal et al., 1986) but not among the different neurotrophins (Hallböök et al., 1991).

The structure of NGF reveals that the monomer forms a flat surface and that the dimer, which is the active form of the NGF molecule, forms by association of two

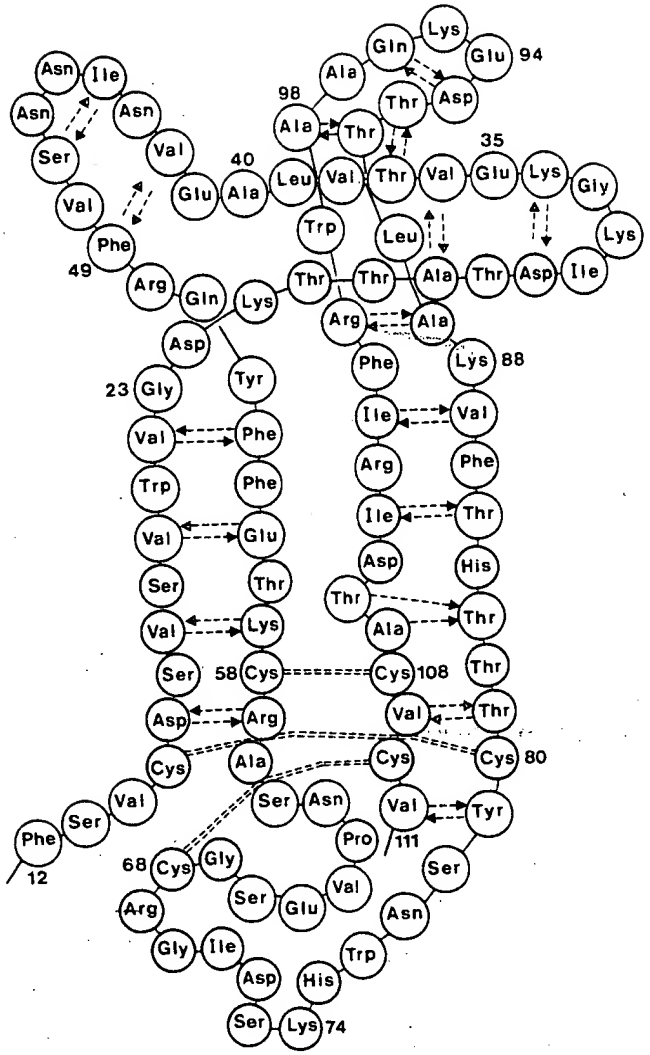

Fig. 2. Schematic illustration of the structure of NGF as revealed by crystallographic data (McDonald et al., 1991). The three disulphide bridges are indicated at the bottom of the figure as are the hydrogen bonds (arrows) involved in the β-sheet structure of NGF. Modified from McDonald et al. (1991).

roughly parallel subunits about a twofold axis via this flat surface. The contacts between the two monomers are mainly hydrophobic and occur via several residues arranged in three groups (McDonald et al., 1991). The majority of well conserved amino acid residues, common to all the known neurotrophins such as Val 36 and 38, Phe 53, Ala 89, Ile 102 and 104, as well as the disulphide bonds (Fig. 2), contribute to a hydrophobic core of the NGF monomer (McDonald et al., 1991). In addition several residues well conserved among the neurotrophins contribute to the structure of NGF by forming hydrogen bonds (Fig. 2). A clustering of positively charged amino

acids, comprising residues Asp 30-Lys 34, has been shown to be important in binding of NGF to the low-affinity receptory NGF receptor (Ibánez et al., 1992).

Fewer than ten amino-terminal and the last few carboxy-terminal amino acid residues are not well resolved by the crystal structure studies (McDonald et al., 1991); it has been suggested that these are flexible and solvent accessible. These parts of the NGF molecule are also known to vary among NGF from different species and to be partially trimmed by proteolytic enzymes without much change in potency of NGF activity.

Thus, it seems very likely that the variable regions, including the three β-hairpin loops that come in close proximity to each other in the NGF protein (Fig. 2), account for the selective high-affinity binding and biological responses in different neurons exerted by the different neurotrophins.

The considerable overall similarity between the different neurotrophins suggests that they all share the revealed NGF structure. The insertion of two extra amino acid residues in BDNF compared with NGF, NT-3, and NT-4/NT-5 occurs before the β-hairpin loop between positions 23 and 24 in the mouse (and rat and human) NGF sequence (Fig. 1). The extra seven amino acids inserted in the human and rat NT-4/NT-5 are also in a loop region, near the reverse turn between Cys 58 and Cys 68 in the mouse NGF. This region is likely to be flexible enough to accomodate such an insertion without disturbing the basic structure of the neurotrophin molecule. Finally, all neurotrophins except NGF found so far have an extra amino acid between positions 94 and 95 in the mature NGF molecule: this insertion (or probably more correctly, this deletion in NGF) occurs in a β-hairpin loop.

RECEPTORS FOR THE NEUROTROPHINS

The neurotrophins exert effects on the responsive neurons by binding to cell surface receptors. The neurotrophic stimulation by NGF is mediated by high-affinity binding sites (K_d of 10^{-11} M; Sutter et al., 1979). Another class of binding sites has a lower affinity (K_d of 10^{-9} M) mediated by a 75 K glycoprotein designated low-affinity NGF receptor (LNGFR) that has been cloned in several species (Johnson et al., 1986; Radeke et al., 1987). Low-affinity binding (K_d of 10^{-9} M) of all tested neurotrophins is mediated via this LNGFR (Rodriguez-Tébar et al., 1990; Ernfors et al., 1990; Hallböök et al., 1991). The function of LNGFR is not well understood at present.

Recently, a 140 K tyrosine protein kinase receptor encoded by the proto-oncogene *trk* has been found to bind NGF with high affinity (K_d of 10^{-11} M) and to evoke the cellular neurotrophic responses (Klein et al., 1991a; Hempstead et al., 1991). In addition, a protein encoded by the *trk*-related gene *trk*B (Klein et al., 1989) has been shown to bind BDNF and NT-3 (Klein et al., 1991b; Soppet et al., 1991; Squinto et al., 1991). Very recently, a third member of the *trk* family, *trk*C, has been cloned and demonstrated to function as a high-affinity receptor for NT-3 (Lamballe et al., 1991).

High-affinity binding to NGF to sections of the adult rat brain was first reported by Richardson et al. (1986). The observations established selective, high-affinity binding of radiolabelled NGF to perikarya probably of cholinergic neurons in the basal forebrain (medial septal nucleus, diagonal band of Broca, lateral preoptic area, and ventrocaudal globus pallidus) and, in addition, to neurons in the caudate-putamen, the medulla oblongata, the ventral cochlear nucleus, and the dorsal nucleus of the lateral lemniscus.

The expression of detectable levels of *trk* and LNGFR mRNA co-localized, and was distinctly restricted to, the medial septal nucleus and the nucleus of Broca's diagonal band (Vazquez and Ebendal, 1991) containing the NGF-responsive magnocellular cholinergic neurons projecting to hippocampus and cerebral cortex.

It is worth noting that *trk* hybridization has also been seen in the striatum (Fig. 3). In these areas, however, there is no corresponding co-expression of LNGFR mRNA (Vazquez and Ebendal, 1991). High-affinity binding of NGF has earlier been demonstrated for scattered neurons in the striatum (Richardson et al., 1986; Fig. 3, right). A particular problem has been to verify the NGF binding in the caudate-putamen (Richardson et al., 1986) with immunohistochemical data for the LNGFR distribution.

Results from in situ hybridization thus show that in the lateral parts of the striatum of the rat, known to bind NGF at high affinity, there is an expression of *trk* mRNA in scattered neurons most likely cholinergic, without a concomitant expression of the LNGFR (Fig. 3).

In sharp contrast, the expression of *trk*B is widespread in many areas of the cortex (piriform, frontal, cingulate) as well as lateral septum and the ependymal lining of the lateral ventricles (Vazquez and Ebendal, 1991; Fig. 4). The neocortex shows labelling with *trk*B in several of the layers (Fig. 4B). The corpus callosum, a structure rich in glial cells, does not exhibit labelling using the oligonucleotide probes.

The most intense labeling in the neocortex using the *trk*B oligonucleotide probe is within layers II, III, and V. Similar patterns of expression have been observed using probes for BDNF (Fig. 4A), a ligand for the *trk*B protein (Soppet et al., 1991; Squinto et al., 1991). This suggests a possible auto- or paracrine function for BDNF in the development and maintenance of the cerebral cortex. Also in other areas of the rat brain, the broad distribution of *trk*B is paralleled by a similar widespread distribution of BDNF mRNA-expressing neurons. It is

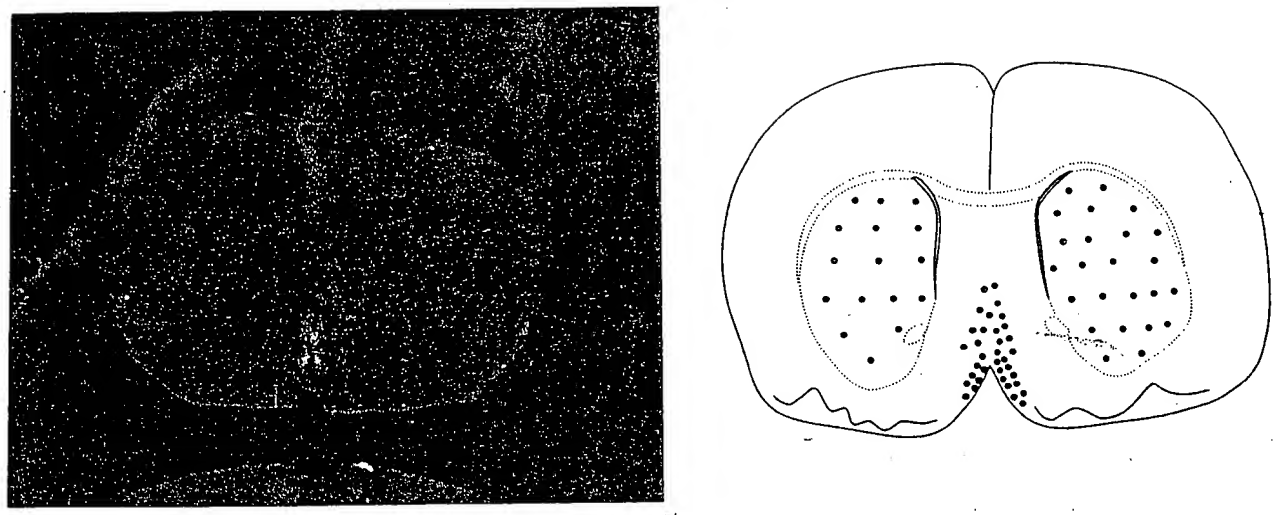

Fig. 3. **Left:** Expression of the high-affinity NGF receptor encoding *trk* mRNA in the rat basal forebrain. Note the strict localization to the septal nucleus and to the diagonal band nucleus, representing labeling of magnocellular cholinergic neurons in these areas. In addition, some scattered cells in the lateral parts of the striatum are labelled, most likely representing striatal cholinergic interneurons. **Right:** For comparison, high-affinity binding pattern for NGF in a similar section, taken from the study of Richardson et al. (1986), is shown.

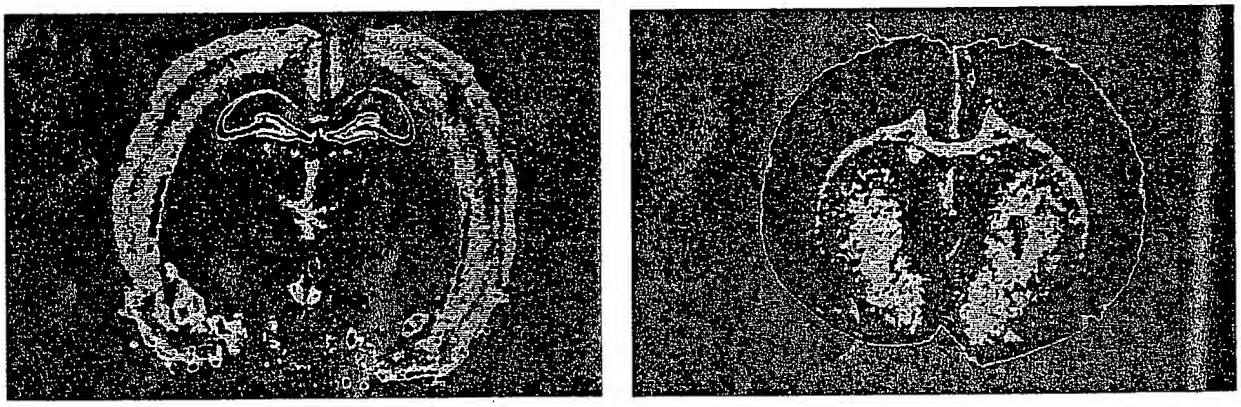

Fig. 4. In situ hybridization of localizing BDNF (**A**) and *trk*B (**B**) in the rat brain. The figure shows coronal sections at the level of hippocampus (A) and striatum (B). The density of labeling is reflected by the pseudocolour code (red representing background signal, to blue representing highest density).

thus apparent that the *trk*B protein might serve general functions in large areas of the cortex.

Although there are obvious preferences for binding of a particular neurotrophin to only one of the *trk*-family of receptors, there is some promiscuity especially if the concentration of neurotrophins is raised (Soppet et al., 1991; Squinto et al., 1991; Lamballe et al., 1991; review by Bothwell, 1991). During evolution it has obviously been advantageous to maintain a common low-affinity receptor for the neurotrophins, while at least three high-affinity receptors confer different but not absolute specificities for their related ligands.

STRUCTURE-FUNCTION RELATIONSHIPS IN THE NGF FAMILY OF PROTEINS

Recent experiments using site-directed mutagenesis and expression of recombinant chimaeric neurotro-

phins, probably representing combinations that have probably never been created during evolution, have demonstrated that particular regions of NGF and BDNF result in the specific actions on different populations of responding neurons. It has also been possible to localize a likely region for the interaction between NGF and the LNGFR. This region could be altered, resulting in the total loss of binding of the mutant NGF protein to the low-affinity NGF receptor without affecting the binding to the high-affinity (*trk*) receptor (Ibáñez et al., 1992).

Selected amino acid residues in the NGF were thus replaced by site-directed mutagenesis (Ibáñez et al., 1990). Mutated NGF sequences were transiently expressed in COS cells and the amount of each mutant polypeptide accumulated in conditioned medium was assessed by protein immunoblotting. The biological activity was determined by measuring stimulation of neurite outgrowth from chick sympathetic ganglia (Ebendal et al., 1991).

The three tryptophan residues at positions 20, 75, and 98 in chicken NGF are all conserved within the family of NGF-like polypeptides (Fig. 1). To define the functional importance of these residues they were replaced with phenylalanine. Both W20F and W98F mutants showed a somewhat reduced activity in the sympathetic ganglion bioassay. The modification of Trp 75 did not significantly affect the activity of the molecule (Ibáñez et al., 1990).

A stretch of aromatic residues around the conserved Tyr 51 has been suggested as a potential site of contact with the NGF receptor. Tyr 51 replaced with phenylalanine gave a similar binding affinity and biological activity as the wild type (Ibáñez et al., 1990).

Ibáñez et al. (1991) studied chimaeric proteins representing different combinations of the variable regions from BDNF replacing the equivalent sequence in the rat NGF. It was possible to show that the introduction of several regions from BDNF did not render the NGF inactive on sympathetic neurons. Rather the additions gradually evoked a response also in the nodose ganglion characteristic of BDNF (and NT-3). Amino acid residues 23–35 including a highly conserved region among NGFs from different species (Ebendal et al., 1986) and the first of the β-hairpin loops of NGF (McDonald et al., 1991) may be involved in the specific NGF-evoked biological stimulation of NGF since the elimination of these two regions by replacement of the corresponding BDNF sequences drastically reduced the fibre outgrowth response in sympathetic ganglia (Ibáñez et al., 1991).

Very recently, Ibáñez et al. (1992) demonstrated that some basic amino acid residues in this region are highly likely to be involved in the binding to the low-affinity receptor for NGF but not so for the high-affinity binding to the *trk* receptor. The binding to the acidic LNGFR protein was thus found to require the basic residues Lys 32, Lys 34, and Lys 95. These are brought in proximity to one another in the NGF protein and may form a surface of positive charge interacting with the LNGFR (McDonald et al., 1991; Ibáñez et al., 1992). Using site-directed mutagenesis to change the basic residues resulted in total lack of binding of the recombinant mutated NGF protein to LNGFR whereas the high-affinity binding and full biological activity in a fibre outgrowth assay with sympathetic ganglia was retained.

The resolution of the crystal structure of NGF revealed a cluster of exposed positively charged side chains close to and around the β-hairpin loop 30–34 (McDonald et al., 1991). It is possible that the overall negative charge LNGFR protein (estimated pI of 4.4; Radeke et al., 1987) forms a basis for an ionic interaction with the highly basic NGF dimer (pI 9.3) in this region (McDonald et al., 1991). The results presented by Ibáñez et al. (1992) provide strong support to the notion that these positively charged amino acid residues serve as the main points of contact between NGF and LNGFR.

PHYLOGENY OF THE NEUROTROPHINS

A cladistic analysis based on sequences encompassing the entire prepro-neurotrophins is presented here. The powerful programme Hennig86 (Farris, 1988) based on parsimony analysis of phylogeny (Farris, 1983; Felsenstein, 1988; Swofford and Olsen, 1990) was used. Although the precursor sequences have some functionally significant conserved parts (Ebendal et al., 1986; Suter et al., 1991), there are substantial differences among species and the different neurotrophins which are not considered to restrict evolutionary alterations. For this reason the precursor sequence was included in the analysis. The phylogenetic tree was rooted by using human proinsulin, a possible ancestor (Frazier et al., 1972; see, however, Argos, 1976), as the outgroup in the comparisons. One of the trees consistent with current views on vertebrate phylogeny (Benton, 1990; Graur et al., 1991) is shown in Figure 5. This tree, like the consensus tree, puts NGF as a sister group to the other neurotrophins. Furthermore, it is indicated that BDNF and NT-4 are closely related and form a sister group with NT-3.

Like the phylogeny presented by Hallböök et al. (1991), the above analysis suggests a radiation of the so far analyzed neurotrophins very early during or even preceeding vertebrate evolution. It is thus worth noting that although NGF or NGF receptors have so far not been described in invertebrates, NGF seems to have effects on neurons from the snail *Lymnaea stagnalis* (Ridgway et al., 1991). Thus, evolution of neurotrophic factors and receptor systems mediating neurotrophic interactions in developmental and neuroplasticity processes may well

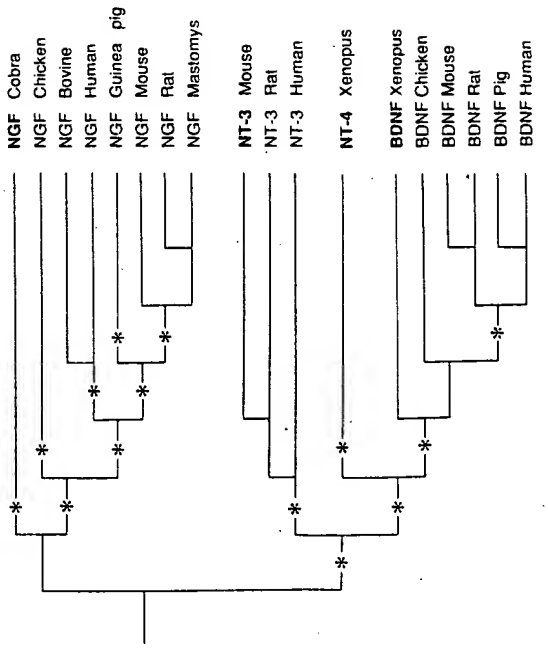

Fig. 5. Phylogenetic tree showing the likely evolutionary relationship between the members of the neurotrophin gene family. The figure is based on an analysis of the prepro-neurotrophin amino-acid sequences using Henning86 computer software (Farris, 1988) and human proinsulin as a hypothetical ancestor (Frazier et al., 1972) to root the tree. NGF from eight different species (mouse, rat, the African rat *Mastomys natalensis*, guinea pig, human, bovine, chicken, and cobra) have been presented by Scott et al. (1983); Ullrich et al. (1983); Ebendal et al. (1986); Meier et al. (1986); Selby et al. (1987a,b); Fahnestock and Bell (1988); Whittemore et al. (1988a,b); Schwarz et al. (1989). The sequences for BDNF and neurotrophin-3 (NT-3) are from Leibrock et al. (1989), Hohn et al. (1990), Maisonpierre et al. (1990), Ernfors et al. (1990a), Kaisho et al. (1990), and Rosenthal et al. (1990), and the *Xenopus* NT-4 sequence is from Hallböök et al. (1991). The heuristic search algorithm of the programme, m*, was used for constructing the initial trees, and the branch breaker feature, bb*, was used to generate multiple parsimonious solutions. The analysis resulted in 180 equally parsimonious phylograms, all of which imply 573 amino acid substitutions, excluding phylogenetically uninformative positions having no substitution or a substitution in a single sequence. One of these trees is shown in the figure. Branches present in all 180 solutions, and hence in the strict concensus tree, are indicated by asterisks. The cladistic analysis showed consistency and retention indices (Farris, 1988, 1989) to be 0.93 and 0.94, respectively, indicating a very low level of homoplasy, i.e., noise, parallel and reversed substitutions comprising only 6–7% in the data.

have begun in ancestors common to several major metazoan phyla. No doubt the flexible system of several neurotrophins with partially overlapping activities combined with several receptors has been adopted to fulfil various functions during vertebrate evolution. It may thus be anticipated that the neurotrophins and their receptors act differently in different groups of vertebrates and also have taken distinct roles in separate organ systems. Accumulating data from studies of neurotrophins not only in the nervous system but in the immume system and in the reproductive organs (Olson et al., 1987; Ayer-LeLievre et al., 1988; Persson et al., 1990) substantiate such a view.

CLINICAL PERSPECTIVES

The rapid progress made in the field of neurotrophic factors has led to an understanding of how these proteins may be of clinical use in situations of neurodegenerative diseases. Magnocellular cholinergic neurons in nucleus basalis of Meynert undergo a profound and selective degeneration in patients with senile dementia of the Alzheimer type (SDAT). A strong correlation also exists between the reduction in cholinergic marker activity and severity of the dementia (Perry et al., 1978; Wilcock et al., 1982). The first clinical trials with NGF infused into the human brain have recently been carried out: One case concerns the use of NGF to support grafted adrenal medullary tissue transplanted to the putamen of a Parkinson patient (Olson et al., 1991), a second case concerns an Alzheimer patient receiving a 3-month period of intraventricular NGF infusion (Olson et al., 1992). In both instances, representing two models of possible future application of NGF therapy, beneficial effects but no ill-effects were documented, encouraging further limited studies in carefully controlled patients.

ACKNOWLEDGMENTS

Kåre Bremer is thanked for valuable discussions on cladistics and for imperative support in the phylogenetic reconstruction data presented here. Stine Söderström and Marcelo Vazquez are thanked for help with in situ hybridizations. I am grateful to Reg Williams for helpful comments on the manuscript. Secretarial help was given by Vibeke Nilsson.

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